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DISTANT TUMOURS PRODUCED BY 2-AMINO- AND 2-ACETYL-AMINO-FLUORENE.

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PATHOLOGISTS and chemists engaged in experimental cancer research look at a new carcinogenic agent from different points of view. The chemist interested in the relationship between chemical structure and biological activity pays attention to any new addition to the long list of carcinogenic compounds. The pathologist's interest is raised if the new substance produces biological effects which are different from the known action of the hydrocarbons or the azo-compounds. Wilson, DeEd and Cox (1914) described the carcinogenic action of 2-acetyl-amino-fluorene. For several reasons this substance deserves the attention of biologists and chemists: (1) It is a compound of very simple chemical structure; (2) when fed it produces a greater variety of tumours in a given strain of rats than most of the known carcinogenic compounds; (3) the localization of the tumours is dependent on the sex of the animal. This last point seems of special interest in view of the varying frequency of certain malignant tumours in men and women.

The purpose of this paper is to give a preliminary report on the types of tumour so far produced by the feeding of 2-acetyl-amino-fluorene to rats. A detailed account of the histological findings will follow later. One experiment dealing with the production of distant tumours by painting the skin of rats with 2-amino-fluorene is included.

Methods.—Albino rats, descendants of Wistar rats, about 2 months old, were used. During the last five years no animals from outside have been added to the colony, which represents therefore a strain of relatively great homogeneity. No spontaneous tumours or leukaemia have been observed during the last four years, nor could tumours of the breast be provoked by "forced breeding." The response of the rats to high doses of oestrogens seems to be the same as described by Geschickter (1943). By following his technique of implanting multiple stilboestrol-pellets under the skin, mammary cancer could be produced.

2-amino-fluorene (M.P. 129) and 2-acetyl-amino-fluorene (M.P. 193) were synthesized according to the method of Diels (1901). The synthesis is simple and the yields are good. Commercial fluorene from different sources was used for the synthesis, and no difference of activity has been observed in the various preparations. It seems

therefore very unlikely that an impurity, present in commercial fluorene and carried through the various steps of synthesis, could be responsible for the biological activity.

The basic diet consisted of 7 g. bread powder and 3 g. skimmed milk powder daily, supplemented by green vegetables and cod-liver oil once a week. When the animals were heavier than 100 g. the amount of food was increased by 20 per cent. In the majority of the experiments acetyl-amino-fluorene was given in a daily dosage of 4 mg. per rat for 30 weeks. Water was added to the food-drug mixture until it had the consistence of porridge ; no extra water was given. The rats consumed their food completely and were unable to pick out the drug. The animals were weighed once a week and examined twice weekly for the presence of tumours. No clinical signs which could be attributed to a toxic action of the drug have been observed with the dosage used, except for the inhibition of growth (Bielschowsky and Green, 1942). If the animals lost weight after 180 to 210 days of daily feeding of 4–6 mg. acetyl-amino-fluorene a tumour was generally present. In some instances infection of the lung or intestine was responsible for the loss of weight and consequent death ; thus in about 10 per cent. of the experimental animals post-mortem examination failed to reveal malignant growth. Some animals became moribund in the course of a few hours without having shown signs of previous illness ; this was due to haemorrhages into the abdomen from tumours situated on the surface of the liver.

Results.—The results presented are based on the post-mortem findings in 104 rats which had received 2-acetyl-amino-fluorene, and of 5 animals which had been painted with 2-amino-fluorene. The latter experiment will be described first. On the assumption that the failure of the American investigators and ourselves to produce local growth by implanting pellets of acetyl-amino-fluorene was due to the incapacity of the peripheral tissues to split off the acetyl group and thus to form the hypothetical active compound, the free amino-fluorene, it was hoped by this method to obtain local tumours. Five 2-months-old male rats were used. The hair of the interscapular region was epilated with BaS, and for a period of 210 days the skin was painted with a 4 per cent. solution of 2-amino-fluorene in acetone thrice weekly. The animals seemed to be completely unaffected by this treatment ; the hair started to grow rapidly again in spite of the painting and the animals grew at the normal rate. 280 days after the beginning of the experiment one rat died from a malignant hepatoma. A careful examination of the four survivors revealed that all had enlarged livers and they were therefore killed. In all four advanced liver tumours were found, and in one of them a carcinoma of the ductus acousticus was also present. Not the slightest alteration in the painted area could be discovered.

Ninety-three of the 104 rats which received acetyl-amino-fluorene per os developed malignant tumours, the localization of which is given in the table.

	Males.	Females.	Castrated males.	Spayed females.	Total. tumours.
Total numbers	41	36	5	11	105
Malignant hepatomas	34	11	5	5	55
Mammary cancer	3	23	..	1	27
Tumour duct. acoust.	5	6	3	2	16
Cancer of intestine	3	..	..	2	5
Tumour of uterus	..	1	..	..	1
Carcinoma of skin	..	1	..	..	..
Leukaemias	2	1	..	1	4

Besides the malignant growths, two types of benign tumour have been observed :

11 rats had benign adenomas in the lungs, and nearly all the animals had more or less pronounced cystic tumours of the liver. These cysts arise from bile-ducts, and they resemble strongly the cystic cholangiomas described by Orr (1940) in rats treated with butter-yellow. In some animals only a few cysts were seen. In others, great areas of the liver were transformed into cystic tissue, which was filled with a clear liquid and looked transparent. Since true invasive growth or metastasis have never been found, the cystic cholangiomas are not included in the list of malignant tumours.

From the table two facts can be deduced: the presence of multiple tumours in several animals (105 malignant tumours in 93 rats), and the unequal distribution of the various types of tumours between the two sexes. Twenty-three of 36 female rats had one or more mammary cancers, but only 3 of the 41 male rats have been observed to have breast tumours. One of the 11 spayed rats developed a cancer of the breast and none of the 5 castrated rats. This unequal distribution is remarkable, since mammary cancers produced by high doses of oestrogens develop in normal and castrated male and in normal and spayed female rats with the same frequency. It seems therefore probable that the pathogenesis of the oestrogen-induced rat tumours is different from the mode of action of 2-acetyl-amino-fluorene. Acetyl-amino-fluorene has no oestrogenic activity; all attempts to obtain a positive Allan-Doisy test by feeding amounts up to 100 mg. for 3 consecutive days failed to provoke oestrus. Nor has oestrus ever been observed in the castrated rats after 200 days of feeding an acetyl-amino-fluorene-containing diet; in such animals the uterus and vagina are atrophic, and the typical changes produced by high doses of sex hormones in adrenals and pituitary are absent. Since the precancerous changes have not yet been investigated, no opinion can be ventured as to how acetyl-amino-fluorene acts on the breast gland, but it must be assumed that the presence of the ovaries is a factor of great importance for the development of mammary cancer in these experiments.

The greater frequency of malignant hepatomas in the male rats is difficult to explain. This phenomenon may be related to the greater sensitivity of male rats to cirrhosis-producing substances or diets. Nodular hypertrophy and increase of connective tissue are frequently observed in male rats kept on an acetyl-amino-fluorene diet. The addition of glycocyamine or cystine in amounts which damage the liver seems to increase the frequency of liver tumours in female rats. Since these substances enhance the tendency to cirrhotic changes and the development of fatty livers, it seems possible that these conditions favour the development of malignant hepatomas.

An unusual type of tumour which was observed in 16 instances is a carcinoma arising from the ductus acousticus externus. It originates in the sebaceous glands of the duct, or starts as malignant papilloma from the squamous epithelium. These tumours are usually heavily infected by the time they can be detected on inspection; they seem to occur in both sexes with about the same frequency.

Five adenocarcinomas of the gut, 3 originating in males and 2 in spayed females, have been discovered during the post-mortem examinations. The small number of cancers of the gut does not warrant an opinion on their frequency in relation to sex and castration. Tumours of the urinary tract which were frequently observed by the American investigators have not been found so far in our material.

The malignancy of the above-mentioned tumours was deduced from their histological structure, their invasive growth, their ability to metastasize, and from transmission experiments. The hepatomas metastasized frequently into the lungs, and one generalized dissemination into the lymph glands has been observed. On 3 occasions carcinomas of the ductus acousticus produced secondaries in the lungs. Only 1 metastasis of a breast tumour has been found, and 1 carcinoma of the caecum metastasized into the liver. The 3 liver tumours which we tried to transplant proved

transmissible ; their growth rate was rather slow. Three cancers of the breast were successfully transplanted ; the first, which originated in a male, produced 100 per cent. takes and grew vigorously in the new hosts. The 2 others, which had developed in female rats, grew at a slower rate ; one of them needed stimulation by oestrogen after the third generation to make it grow in immature rats.

One leukaemia which originated in a spayed rat was discovered early enough to allow transmission experiments. The rat was bled and the necropsy, performed immediately, did not reveal the presence of a tumour besides the state of leukaemia. Three young animals were injected subcutaneously with 0·5 ml. of the blood, and in all three rapidly growing tumours developed after two weeks at the site of the injection. None of the injected rats showed any changes in the number or type of leucocytes for the first three weeks, but a leukaemic blood-picture appeared later. Intravenous injection of such blood or of a suspension of tumour-cells from the subcutaneous tumour invariably produced leukaemia in fresh animals, most of which showed small local tumours at the site of the injection. In one a very careful examination failed to reveal tumour tissue around the injected vein. These " secondary " leukaemias were again successfully transmitted by intravenous injection.

The great majority of the above-mentioned tumours and all the leukaemias were discovered from 180 to 280 days after the beginning of the experiment. The earliest tumour seen so far was a mammary cancer observed at the 99th day. The earliest hepatoma and carcinoma of the ductus acousticus were found at the 147th and 154th day respectively. A few tumours appeared later than 42 weeks.

SUMMARY.

The production of distant tumours by 2-amino-fluorene and 2-acetyl-amino-fluorene is described. The results obtained by Wilson, DeEd and Cox with 2-acetyl-amino-fluorene have been confirmed. The influence of the sex organs on the localization of some of the 2-acetyl-amino-fluorene-produced tumours is demonstrated. The malignancy of the hepatomas and the tumours of the breast and the true leukaemic character of the blood changes observed in one spayed rat have been stablished by transmission.

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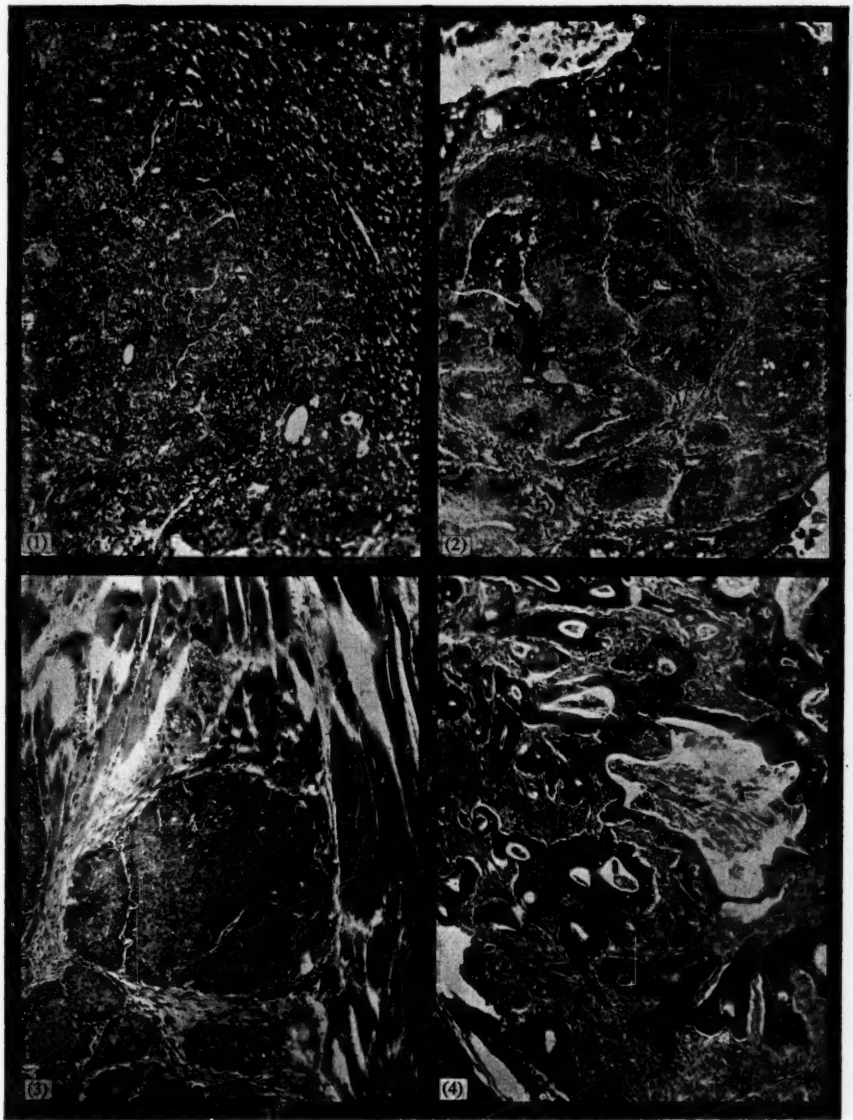

FIG. 1.—Hepatoma produced by painting the skin with 2-amino-fluorene.
FIG. 2.—Cancer of the breast.
FIG. 3.—Carcinoma of ductus acousticus externus.
FIG. 4.—Adenocarcinoma of the small intestine.
Lesions shown in Figs. 2–4 produced by feeding of 2-acetyl-amino-fluorene.

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THE OCCURRENCE OF A, B AND O BLOOD GROUP SUBSTANCES IN PSEUDO-MUCINOUS OVARIAN CYST FLUIDS.

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THE difficulty of obtaining sufficient group specific substance from human erythrocytes for detailed immunochemical studies forced us to consider human tissue fluids and secretions as a source of these factors. The capacity of certain individuals to secrete in their tissue fluids substances that possess a serological specificity very similar to or identical with their specific blood group factors is now generally recognized, and on the basis of the earlier work of Yamakami (1926), Greenfield (1928), Yosida (1928), Brahn and Schiff (1929), Lehrs (1930), Schiff (1931), and of Witebsky and Klendshoj (1940, 1941) it appeared probable that either gastric juice or saliva would be the most potent and readily available source of the specific substances. Lehrs (1930) and Putkonen (1930), however, found that whereas the saliva of certain individuals who belonged to groups A, B and AB contained the specific blood group factor, the saliva of certain other individuals of the same blood group was almost free from the specific substance. Schiff and Sasaki (1932) and Sasaki (1932) have designated those individuals who secrete the blood group factor in their saliva "secretors," whereas those who do not secrete the active factors are termed "non-secretors." Somewhat later Schiff (1934) showed that persons belonging to group O could likewise be subdivided into secretors and non-secretors. It is now generally accepted that the presence or absence of water-soluble specific blood group factors in the tissue fluids of an individual is controlled by a single gene S, dominant in effect, which allows the secretions of the specific substance. According to Friedenreich and Hartmann (1938) and Hartmann (1941) the S gene is to be considered as a complementary factor, the presence of which is necessary for the development and secretion of the water-soluble form of the blood group substance. There is no evidence of linkage between the character "secretor" and any of the A, B, O blood groups (Wiener, 1943).

The concentration of the active factors in saliva and gastric juice is undoubtedly high when compared with other tissue fluids, but even here the active factor represents only a small part of the total solid matter in the secretions, which are moreover difficult to obtain in large quantities. In certain instances, however, mucilaginous fluids accumulate in man as a result of pathological changes or of tissue overgrowth, and the knowledge that pseudo-mucinous ovarian cysts are of frequent occurrence prompted us to examine the fluid contents of these pathological overgrowths for blood group factors. The first fluid examined was active in inhibiting A specific iso-agglutination after diluting several thousand times, and the volume of cyst fluid in that instance was rather more than 3 litres. After the examination of further cyst fluids it became

evident that pseudo-mucinous ovarian cyst fluids were a potent and convenient source of blood group substances. Later in the investigation it was found that we had overlooked an earlier observation of Yosida (1928), who recorded that of three ovarian cyst fluids he had examined two contained blood group factors. No further details were given.

The following account is a record of the examination of 50 pseudo-mucinous cyst fluids for the blood group factors A, B, O, and in some instances for Rh. In most examinations the saliva of the patient was titrated for specific substance in the same experiment as the cyst fluid. By this means it was possible to correlate the capacity of the person to secrete the blood group substance in the saliva with the occurrence of the active factor in the cyst fluid.

EXPERIMENTAL.

Pseudo-mucinous ovarian cyst fluids.—The cyst fluids were collected from hospitals as soon as possible after removal from the patient. Toluene was added as preservative. For the quantitative determination of the blood group factor in the fluid a portion of the fluid from each patient was spun to remove tissue debris, frozen at −20° and dried *in vacuo*.

The cyst fluids varied in volume from about 100 ml. to nearly 7 litres. Cyst fluids that contained a high concentration of the A factor were frequently found to be pale yellow, clear, viscous solutions. The total solids were at times as high as 16 per cent., but the most active group A cyst fluid encountered, from Case 28, contained only 5·6 per cent. solid matter. All the cyst fluids gave heavy precipitates with trichloroacetic acid and sulpho-salicylic acid, showed a strong laevo-rotation and contained between 7 and 13 per cent. N. The active B and O cyst fluids contained much less specific blood-group substance than the A fluids, and in consequence it was not possible with these specimens to differentiate the active from the inactive cyst fluids by merely observing the relative viscosity.

Blood and saliva.—Specimens were collected from each patient. The saliva was preserved with toluene.

Anti A- (α) *and anti B-* (β) *agglutinin.*—Pooled samples of serum collected from persons of groups B and A respectively were used throughout the investigation. The sterile serum which was of high potency against A_1 and A_2 cells was stored in small (1–2 ml.) ampoules at −20° without the addition of antiseptic. A fresh ampoule of serum was opened for each series of tests, and the sera were tested at frequent intervals during the investigation for α- and β-agglutinins active against the erythrocytes of selected group A and B persons working in the laboratory. The final dilution of anti-A and anti-B serum used in the inhibition tests showed an agglutination titre of 1 : 192 against A_1 and B cells respectively.

Anti-O agglutination.—Serum samples collected from 52 normal cattle were examined for O agglutinins after they had been exhausted of anti-A (α) and anti-B (β) agglutinins and of human species immune-body by absorption with fresh A_1B erythrocytes, according to the method described by Schiff (1927), Sasaki (1932), and Witebsky and Klendshoj (1941). Six of the sera showed useful O agglutinins, the three most potent of which were pooled and the sterile mixed sample used as a standard throughout the investigation. Just before use the serum was diluted with four volumes of saline and absorbed with fresh A_1B cells. The reactions of a typical anti-O reagent prepared in this manner are shown in Table I. The strength of the O agglutination resembled the weak Rh agglutination rather than the strong clumping given by A and B cells with their homologous immune-body.

TABLE I.—*Showing the Agglutination of A_1, B, A_1B and O Cells with Absorbed Cattle Serum.*

Test cells.	Dilution of serum showing agglutination.				
	1 : 5.	1 : 10.	1 : 20.	1 : 40.	1 : 80.
O . .	3	3	2	1	1
A_1B . .	0	0	0	0	0
A_1 . .	1	0	0	0	0
,, . .	0	0	0	0	0
B . .	1	0	0	0	0

Degrees of agglutination : 0, no agglutination ; 1, a few small groups of cells ; 2, larger groups with some free cells ; 3, strong agglutination.

Inhibition tests.—The detection and quantitative measurement of the specific A, B and O substances present in the different cyst fluids was determined by the method of inhibition of iso-agglutination. The immune-serum, anti-A (α), anti-B (β) or absorbed anti-O (0·1 ml.) was run into small test-tubes from standard pipettes. Each 0·1 ml. of anti-A and anti-B serum contained 3–4 completely agglutinating doses of agglutinin. The test dose of O-agglutinin on the other hand represented about two strongly agglutinating doses only. This amount of O-agglutinin allowed the agglutinated cells to be readily dispersed by gentle shaking, and in no way resembled the tight, firm agglutination given by 3–4 completely agglutinating doses of anti-A and anti-B with A and B cells respectively. Serial dilutions of cyst fluids (0·1 ml.) were then added to each tube, and after thorough shaking the mixtures were allowed to stand at room temperature for an hour, after which time a 0·5 per cent. suspension of freshly drawn A or B erythrocytes (0·1 ml.) in sterile saline was added ; the contents of the tubes were then thoroughly mixed and set aside for 2 hours at room temperature. The degree of A and B iso-agglutination for those tubes that showed weak agglutination was observed under the low power of the microscope. In order to avoid undue dilution of the O-anti-O system the washed O cells were added as a 2·5 per cent. suspension (0·02 ml.). The extent of O agglutination was observed microscopically, using a gentle technique for resuspending the agglutinated cells similar to that employed for reading Rh agglutination (Taylor, Race, Prior and Ikin, 1942).

The Examination of Cyst Fluids for Blood Group Specific Substances.

Qualitative examination.—A preliminary examination of the cyst fluids and saliva for specific blood group substances was carried out after the specimens had been cleared of any insoluble tissue debris by centrifugation. These preliminary observations, in which simple progressive double dilutions of the native cyst fluid and saliva were used in inhibition tests, enabled the specimens to be roughly classified as active or inactive, and the patients to be differentiated into secretors or non-secretor types. In Table II are set out the results obtained in inhibition tests using cyst fluids and the corresponding salivas from patients of group A, B and O.

During the early stages of the investigation attempts were made to measure the O substance by titration against an anti-O serum prepared in the rabbit according to the technique described by Hooker and Anderson (1921) and by Matta (1937). The sera after absorption with A_1BMN cells gave reagents that reacted only weakly with O erythrocytes, and failed to differentiate sharply between secretors and non-secretors belonging to group O. The use of absorbed cattle serum overcame this difficulty, but the earlier saliva samples were then no longer available for re-test. In order to

TABLE II.—*The Examination of Pseudo-mucinous Ovarian Cyst Fluids for A, B and O Blood Group Substances.*

Case number.	Blood group.	A, B or O agglutination inhibited by—		Case number.	Blood group.	A, B or O agglutination inhibited by—	
		Cyst fluid.	Saliva.			Cyst fluid.	Saliva.
1	A	+	+	26	A	0	0
2	B	0	0	27	B	+	+
3	O	+	+*	28	A	+	+
4	O	—	—	29	AB	0	0
5	O	—	—	30	A	+	+
6	A	0	0	31	O	+	+
7	O	0	0*	32	O	0	0
8	O	0	0*	33	O	0	0
9	O	0	0*	34	A	0	?
10	A	0	0	35	A	+	+
11	A	0	0	36	A	0	0
12	O	—	—	37	A	+	+
13	O	—	—	38	O	0	0
14	A	0	0	39	O	0	0
15	O	+	+	40	O	0	0
16	O	+	+	41	O	0	0
17	O	0	0	42	O	0	0
18	O	0	0	43	A	0	0
19	A	+	+	44	O	0	0
20	A	+	+	45	O	+	+
21	B	+	+	46	O	0	0
22	O	0	0	47	O	0	0
23	O	0	0	48	A	+	+
24	O	0	0	49	A	0	0
25	O	0	0	50	O	0	0

+, specimen showed power to inhibit the corresponding agglutinin; 0, specimen showed no power to inhibit the corresponding agglutinin; —, not tested.

* Saliva tested against anti-O rabbit serum.

complete Table II, the results of the inhibition tests with the absorbed rabbit serum and the early saliva specimens have been recorded.

The results given in Table II should not be used to estimate the frequency of the secreting types, because the crude cyst fluids and salivas have not been recorded as active unless they have given complete inhibition at dilutions greater than 1 : 4 or 1 : 8. Partial inhibition at these dilutions has sometimes occurred but has been recorded as "inactive." It is evident from the results of these qualitative tests that the cyst fluids frequently contain the same specific blood group factor as occurs in the erythrocytes and saliva, and it is of interest to note that in no instance does the specific blood group factor occur in the cyst fluid if it is absent from the saliva and *vice versa*.

Quantitative examination.—In order to obtain an approximate idea of the absolute amount of the A, B and O substances present in the cyst fluids, the activity of the native fluid has been compared with that of known active salivas on a dry weight basis. A specimen of each fluid was frozen at −20° and dried *in vacuo* from the frozen state. A 1 per cent. or 0·1 per cent. solution of the dry material was then made in normal saline and the inhibiting activity of serial dilutions of the solutions was

determined. The results of tests with cyst fluid substance from persons of group A, B or O are set out in Tables III, IV and V respectively. Each table includes the results obtained with two samples of saliva from secretors of the appropriate blood group. In each instance the saliva specimens are the most potent we have encountered in the course of the examination of a large number of samples. In Table III a preparation of the A active polysaccharide-amino acid complex isolated by Morgan and King (1943) from hog gastric mucin has been included for comparison.

TABLE III.—*The Amount of Specific Blood Group A Substance in Pseudo-mucinous Ovarian Cyst Fluids from Group A Persons.*

Case number.	Total volume of cyst fluid (ml.).	Dilution of dry cyst substance inhibiting anti-A (α) agglutinin.									
		1:10^3.	1:10^4.	1:4×10^4.	1:8×10^4.	1:16×10^4.	1:32×10^4.	1:64×10^4.	1:128×10^4.	1:256×10^4.	1:512×10^4.
1	3090	0	0	0	1	2	3	4	4	4	4
19	1600	0	0	0	0	0	1	2	3	4	4
28	500	0	0	0	0	0	0	1	2	3	4
35	320	0	0	0	0	0	1	2	3	4	4
37	2500	0	0	0	0	0	1	2	2	3	4
48	2100	0	0	0	0	0	0	1	2	2	4
90% phenol insoluble fraction from cyst 19		0	0	0	0	0	0	0	0	1	2
"A" substance from hog gastric mucin		0	0	0	0	0	0	1	2	3	4
Saliva 1		0	0	1	2	3	4	4	..	..	..
„ 2		0	0	0	1	2	3	4	..	..	..

Degrees of agglutination: 0, no agglutination; 1, a few groups of 2–3 cells; 2, larger groups with many free cells; 3, small clumps visible to the naked eye; 4, massive agglutination.

The cyst fluids from Cases 28 and 48 (Table III) are the most active in the series of A cysts, and the dry cyst substances give complete inhibition of A-iso-agglutination at a dilution of $1 : 32 \times 10^4$. In this instance the crude cyst substance is therefore as active as the purest A substance that was isolated from hog gastric mucin, and is many times more active than the most potent group A salivas we have encountered, which inhibit completely under the same conditions at a maximum dilution of $1 : 40 \times 10^3$. The crude cyst substances can be purified by a technique similar to that developed by Morgan and King (1943), and a polysaccharide complex showing an enhanced activity in inhibition tests can readily be isolated. For example, the material from cyst 19 that was insoluble in 90 per cent. phenol was found to be several times as active as the crude cyst substance, and the greatest dilution showing complete inhibition of agglutination under the test conditions described was $1 : 128 \times 10^4$. A detailed account of the isolation and properties of the specific A, B and O substances from pseudo-mucinous cyst fluids will be given elsewhere. The results given in Tables III, IV and V were obtained in single experiments using the same dilution of anti-A, anti-B or anti-O agglutinin and the same suspension of A_1, B or O erythrocytes respectively throughout each experiment; the results within each Table are therefore strictly comparable.

In the series of 50 ovarian cyst fluids given in Table II only three belonged to group B and one of these was inactive. The most active cyst fluid in the series (Table IV, Case 21) gave complete inhibition of iso-agglutination at a dilution of $1 : 128 \times 10^2$, and was somewhat less active than the best saliva specimen tested which gave complete inhibition at a dilution of $1 : 256 \times 10^2$.

TABLE IV.—*Showing the Amount of Specific Blood Group B Substance in Pseudo-mucinous Ovarian Cyst Fluids from Group B Persons.*

Case number.	Total volume of cyst fluid (ml.).	Dilution of dry cyst substance inhibiting anti-B (β) agglutinin. 1 : 2×10².	1 : 4×10².	1 : 8×10².	1 : 16×10².	1 : 32×10².	1 : 64×10².	1 : 128×10².	1 : 256×10².	1 : 512×10².	1 : 1024×10².
2	..	4	4	4	4	4	4	4	4	..	..
21	400	0	0	0	0	0	0	0	1	2	2
27	1060	0	0	0	0	0	1	2	3	4	4
Saliva 1	. . . .	0	0	0	0	0	0	0	0	1	2
„ 2	. . . .	0	0	0	0	0	0	0	1	2	3
„ 3	. . . .	0	0	0	1	2	3	3	4	4	4

Degrees of agglutination as in Table III.

The most potent O cyst fluid (Table V, Case 3) completely inhibited the agglutinating action of anti-O cattle serum at a dilution of $1 : 512 \times 10^2$. The O substance in this instance is about four times as potent as the most active O salivas we have examined (Table V, 1 and 2).

TABLE V.—*Showing the Amount of Specific Blood Group O Substance in Pseudo-mucinous Ovarian Cyst Fluids from Group O Persons.*

Case number.	Total volume of cyst fluid (ml.).	Dilution of dry cyst substance inhibiting cattle anti-O agglutinin. 1 : 2×1².	1 : 4×10².	1 : 8×10².	1 : 16×10².	1 : 32×10².	1 : 64×10².	1 : 128×10².	1 : 256×10².	1 : 512×10².	1 : 1024×10².
3	1150	0	0	0	0	0	0	0	0	0	1
8	..	2	3	3	3	3	3	3	3	3	3
15	760	0	0	0	0	0	1	2	2	3	3
16	1200	0	0	0	0	0	1	2	2	2	3
31	..	0	0	0	0	0	1	2	2	3	3
45	300	0	0	0	0	0	0	0	0	1	2
Saliva 1	. . . .	0	0	0	0	0	0	0	1	2	2
„ 2	. . . .	0	0	0	0	0	0	0	1	1	2

Degrees of agglutination as in Table I.

TABLE VI.—*The Agglutination of O Erythrocytes by Absorbed Anti-O Cattle Serum after Treatment of the Latter with Pseudo-mucinous Ovarian Cyst Fluids containing A, B or O Specific Substances.*

Cyst number.	Blood group.	Secretor (S) or non-secretor (s).	Degree of agglutination. Dilution of cyst substance. (× 100.) 1 : 1.	1 : 2.	1 : 4.	1 : 8.	1 : 16.	1 : 32.	1 : 64.	1 : 128.	1 : 256.	1 : 512.	1 : 1024.	1 : 2048.
1	A	S	1	0	0	0	0	0	1	1	2	2	3	3
19	„	„	2	2	3	3	3	3	..	..	..	..	..	..
28	„	„	2	2	2	2	2	2	3	3	3	3	3	3
37	„	„	1	2	2	3	3	3	..	..	..	..	..	..
43	„	s	2	3	3	3	3	3	3	3	3	3	3	3
21	B	S	2	2	3	3	3	3	..	..	..	..	..	..
3	O	„	1	0	0	0	0	0	0	0	0	0	1	1
45	„	„	1	0	0	0	0	0	0	0	0	1	2	2
8	„	s	2	2	3	3	3	..	..	..	..	..	..	..
Crude gastric mucin (Wilson)			1	1	0	0	0	0	0	0	0	1	2	2
"A" substance from crude gastric mucin (Wilson)			3	2	1	0	0	0	0	0	0	1	2	2
Partially purified "A" substance from cyst No. 1			1	1	0	0	0	0	0	1	1	2	2	2

Degrees of agglutination as in Table I.

The Influence of A and B Substances on the Agglutination of O Cells by Anti-O Cattle Serum.

It has been recorded by Sasaki (1932), Schiff and Sasaki (1932) and by Witebski and Klendshoj (1941) that saliva and gastric juice obtained from secretors belonging to groups A and B inhibit the action of cattle anti-O agglutinin on human O cells. We have, therefore, investigated the inhibiting action of the active cyst fluids on the agglutination of O erythrocytes by absorbed cattle serum. The cyst fluids chosen belonged to group A, Cases 1, 19, 28, 37 and 43, and to group B, fluid from Patient 21. The cyst fluid from a non-secretor of group O (Case 8) and active fluids of the same group (Cases 3 and 45) were included as controls. In each instance amounts of the cyst substance (0·1 ml.) consisting of progressively doubled dilutions of a 1 per cent. solution of dry cyst substance were mixed with 0·1 ml. of the anti-O cattle serum. After standing at room temperature for an hour, 0·02 ml. of freshly drawn and washed 2·5 per cent. O cells were added and thoroughly mixed. The degree of agglutination was read after two hours. The only cyst fluid which gave rise to inhibition of agglutination apart from Nos. 3 and 45, which were known to be potent samples of O specific substance, was cyst fluid No. 1, which belonged to group A. No inhibition of O agglutination was observed with the other six A or B active or inactive cyst fluids. The results are set out in Table VI. A partially purified specimen of cyst 1 that was insoluble in 90 per cent. phenol and which showed an enhanced A activity was found to inhibit O agglutination in much the same way as the crude cyst substance. Purified A substance isolated from pig gastric mucin (Morgan and King, 1943) showed partial inhibition out to a dilution of 1 : 400 and complete inhibition from this dilution to 1 : 25,600. The purified A substance was, therefore, almost as active in neutralizing anti-O agglutinins as was the most potent O cyst fluid. The commercial peptones " Witte " and " Difco " showed no power to inhibit O-agglutination at dilutions above 1 : 100, whereas a specimen of pepsin (Parke Davis, 1 : 10,000) gave rise to complete inhibition up to a dilution of 1 : 800. Very similar results were obtained using another specimen of cattle serum absorbed with A_1B cells from the same donor. On the other hand, with other specimens of absorbed anti-O cattle serum, cyst substance 1 showed a partial inhibition only at all dilutions between 1 : 100 and 1 : 6400. In a set of experiments employing the same absorbed anti-O cattle serum and in which A and B salivas from secretors and non-secretors replaced the cyst fluid, we were able to confirm in nearly all instances the earlier findings of Sasaki (1932) and Witebsky and Klendshoj (1941). A total of six group A_1 salivas, three group A_2, four group B and three group A_1B salivas were tested. Two specimens gave unexpected results: a saliva from a group A non-secretor gave complete inhibition up to a dilution of 1 : 800 of the dry saliva substance, whereas a saliva from one group B secretor failed to show any significant inhibition of the O-anti-O system even at a dilution of 1 : 100. The tests with these samples were repeated several times with the same results. We have not encountered a sample of crude A or B saliva substance that inhibits completely O agglutination at a dilution greater than 1 : 800, whereas the four samples of active O saliva described in Table V give complete inhibition under the same test conditions at dilutions of 1 : 51,200, 1 : 3200, 1 : 3200 and 1 : 25,600 respectively.

Iso-agglutinins in Cyst Fluids.

It has frequently been shown that tissue fluids other than serum can contain iso-agglutinins. We have examined a number of cyst fluids obtained from patients of different blood groups and classified as secretors or non-secretors. The tests showed, for example, that the crude cyst fluid from Case 19, which is rich in blood group A sub-

stance, contains β-iso-agglutinins. Similarly the cyst fluid from Case 21, a group B secretor, contained α-agglutinins only, whereas both α- and β-iso-agglutinins are contained in cyst fluid 3, obtained from a group O secretor. Patients 17 and 44 were non-secretors belonging to group O, and the cyst fluids from these women both contained quite strong α- and β-iso-agglutinins. These results confirm the earlier findings of Cavalieri (1922) and of Weil and Wall (1925).

Rh Substances in Cyst Fluid.

Although at the time this work was started there was some evidence that the Rh factor did not occur in tissue fluids and secretions, it was considered of sufficient importance to test the cyst fluids from patients containing the Rh factor in their erythrocytes to determine if Rh substance was secreted in a water-soluble form. Serial dilutions (1 : 100 to 1 : 1600) of the cyst fluids (0·1 ml.) were mixed with 0·1 ml. of a potent anti-Rh serum that was free from anti-A (α) and anti-B (β) agglutinins, and allowed to stand at room temperature for one hour. Erythrocytes belonging to group O Rh were then added and the mixtures were incubated at 37° for two hours. Cyst fluids from A, B, O non-secretors were included if the patients' erythrocytes contained the Rh agglutinogen. In no instance was any inhibition of the Rh-anti-Rh system brought about by the cyst fluids.

Haemolysis Inhibition Tests.

The results of haemolysis inhibition tests, set up as described earlier (Morgan, 1943), showed that the crude cyst substance from Patient 28 was a more potent inhibitor of the lytic action of an anti-A rabbit serum on sheep red cells than was the purest sample of A substance obtained from hog gastric mucin (Morgan and King, 1943). The purified A substance isolated from cyst fluid 19 likewise showed an enhanced power to inhibit the haemolysis of sheep cells, and a partial inhibition of lysis was readily demonstrable with as little as 0·0005μg. of the purified material.

DISCUSSION.

One of the main objects of the investigation was to discover a more potent and readily available source of water-soluble blood group substances (A, B, O) than saliva or gastric juice, secretions that have generally been considered to be the most potent sources of blood group substances within the human body. In our experience it is difficult to obtain quantities of saliva greater than 5 or 10 ml. per day from persons not directly interested in the experiment, although willing collaborators sometimes produce as much as 40–50 ml. in this period. The preservation of native saliva during its collection, usually at room temperature and over a number of days, is difficult owing to the heavy bacterial contamination originally present. Furthermore, the presence and action of the " Blutgruppenferment " of Schiff and Weiler (1931*a* and *b*) must be considered, as it represents a possible cause of destruction of specific substances that cannot be readily assessed. Although these technical difficulties can to some extent be overcome, as we shall describe elsewhere, the fact remains that the collection of sufficient serologically active saliva or gastric juice to enable a thorough physical and chemical examination of the blood group factor to be carried out is at best a tedious procedure, and all too often becomes a thoroughly objectionable routine for the donor when large quantities of the secretions are required. The amount of active material recovered from saliva or gastric juice is small. For example, Landsteiner (1936) isolated 5 mg. of active A substance from 1 litre of horse saliva and an average

of 15 mg. of A, B and O substance from 500 ml. portions of human saliva. Witebsky and Klendshoj (1940, 1941) record the isolation from gastric juice of 13·5 mg. of B substance and 185 mg. of O substance from 120 ml. and 1 litre respectively of the secretion.

Evidence is now given that pseudo-mucinous ovarian cyst fluids obtained from persons possessing the power to secrete in their saliva the blood group factors in a water-soluble form frequently contain these specific substances in large amounts. Pseudo-mucinous ovarian cyst fluids are, furthermore, of frequent occurrence, are sterile at the time of removal from the patient, are obtained in most instances in very useful quantities and show considerable activity. The group A cyst fluids from Cases 19 and 37, for example, measured 1600 ml. and 2500 ml. respectively, were clear, viscous solutions, and inhibited completely A-iso-agglutination at dilutions of the native fluid of $1 : 80 \times 10^3$ and $1 : 40 \times 10^3$. The patients' salivas, on the other hand, inhibited iso-agglutination, under the same conditions, at a dilution of 1 : 128 and 1 : 256 only, whereas the saliva of two other A secretors, selected because of the high potency of their saliva, gave inhibition at dilutions no higher than 1 : 512 and 1 : 256 respectively. The quantitative data presented show that cyst fluids may contain as much as a hundred times as much specific blood-group A factor per ml. of native cyst fluid as is contained in the same quantity of a good specimen of active A saliva.

The number of cyst fluids examined from patients of group B that secrete this factor is small, and may account for the fact that a really potent anti-B fluid was not encountered. It seems probable that the examination of other samples of cyst fluid from B secretors will reveal the presence of much more potent fluids than the two specimens described. It is to be noted, however, that in each instance the potency of the cyst fluid was similar to that of a good specimen of active saliva. As the combined specimens of B fluid measured about 1·5 litres, it was considered to be a useful amount of starting material for the isolation of B substance.

The specimens of cyst fluid from secretors belonging to blood group O so far obtained are somewhat more active than O salivas, and definitely weaker in content of specific factor than certain of the group A cyst fluids. A more accurate comparison of the potency of the different types of fluid is not possible, owing to the relatively weak agglutination of O cells by anti-O serum when compared with the intense clumping shown by A and B erythrocytes and their corresponding iso-agglutinins.

The high concentration of the A, B and O factors found in the cyst fluids suggested that these secretions should be carefully examined for Rh substance. Since it was possible that the secretion of the Rh factor was controlled by a gene other than the S gene which determines the secretion of water-soluble A, B and O substances, tests for Rh factors in the cyst fluids of Rh patients included those obtained from A, B and O non-secretors. In no instance was there any inhibition of the Rh agglutination observed, thus indicating the absence of water-soluble Rh substance. These results fall into line with those of Wiener and Forer (1941), Levine and Katzin (1941) and Boorman and Dodd (1943) using other tissues and tissue fluids. These workers believe that the Rh factor is not secreted in a water-soluble form.

It is of considerable interest to find that whereas the A or B saliva substance obtained from secretors inhibits the action of absorbed anti-O cattle serum on human O erythrocytes, cyst-fluids that contain potent A or B substances generally cause no inhibition of O agglutination. On the basis of the results obtained with active A and B saliva and gastric juice it might be assumed (see Witebsky and Klendshoj, 1941) that the A and B specific structures each possess some measure of O specificity, and such an assumption receives support from the fact that the undegraded and exceedingly active A substance isolated from hog gastric mucin also shows powerful O inhibiting

properties. Indeed, this material inhibits O agglutination almost as well as the most active crude cyst fluid substance obtained from an O secretor (Case 3, Table V). Moreover, it is to be noted that carefully purified A and B substances obtained from saliva and gastric mucin still retain the capacity to inhibit the action of O agglutinins on O erythrocytes. In view of the considerable O activity shown by the purified A polysaccharide of hog gastric mucin, it is of interest that the artificial antigen made from this substance (Morgan, 1943) gives rise to potent anti-A (α) agglutinin, and yet fails completely to induce in the rabbit the formation of anti-O immune-body. The assumption that the A and B specific substances possess a common and essential structure that is responsible for the O reactivity seems improbable, however, when one considers that the A and B substances in crude cyst fluid and in the purified homologous A polysaccharide, which is the most powerful A substance we have encountered from any source, gives rise to no inhibition of the agglutination of O erythrocytes by an absorbed anti-O cattle serum. An explanation of the mechanism of these serological cross-reactions based on a more exact knowledge of the chemical structures concerned must await the results of a closer examination of the purified and homogeneous A and B substances from saliva and cyst fluid.

Some preliminary experiments have shown the purified specimen of A substance prepared from cyst fluid 19 to be without antigenicity, and a group of rabbits after receiving intravenously six doses each of 0·1 mg. of the A substance showed no immune response. An artificial antigen made from this sample of A substance according to a method already described (Morgan, 1943) gave rise to potent anti-A (α) immune-body in the same animals as had previously received the unconjugated A-substance. The pooled serum agglutinated A erythrocytes at a dilution of 1 : 12,800, whereas B and O cells were agglutinated by dilutions of the serum not significantly greater than those required before immunization.

SUMMARY.

(1) Pseudo-mucinous ovarian cyst fluids obtained from women that possess the ability to secrete their specific blood group substance in a water-soluble form are a convenient and potent source of the group specific substances A, B and O.

(2) In no instance was the blood group character of the ovarian cyst fluid at variance with the specificity of the water-soluble group antigen in the patient's saliva.

(3) Whereas the majority of salivas obtained from secretors belonging to groups A and B inhibit the agglutination of O erythrocytes by absorbed anti-O cattle serum, only one of several specimens of active A or B cyst fluid was found to give rise to any significant inhibition of O agglutination.

(4) Cyst fluids obtained from secretors belonging to groups A, B or O frequently contain anti-B, anti-A or anti-A and anti-B agglutinins respectively.

(5) Water-soluble Rh substance is not present in cyst fluids obtained from secretors and non-secretors belonging to groups ARh, BRh and ORh.

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THE SERUM COLLOIDAL GOLD REACTION AS A LIVER FUNCTION TEST.

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SINCE the report by Yezler in 1930 of the results obtained in cases of liver disease by using the Takata-Ara (1925) reaction on serum two other flocculation tests of liver function have been introduced. These are the cephalin-cholesterol test of Hanger (1939) and the serum colloidal gold reaction of Gray (1940). They appear to be similar in principle, but are said to be both more sensitive and more specific than the Takata-Ara reaction, avoiding for example the " false positive " results given with this test in nephritis. All three tests probably depend upon changes in the serum globulins which are not revealed by ordinary salting-out procedures, certain types of globulin having the power to precipitate negatively charged colloidal particles in the presence of serum albumin which exerts a protective influence on the colloid. Recent work on the electrophoresis of the serum proteins (Gray and Barron, 1943 ; Kabat *et al.*, 1943) suggests that the gamma globulin is the most important fraction, but one difficulty which has not so far been considered is that even the gamma globulin, with an isoelectric point at about pH 6·0, would be negatively charged above this pH, and yet it is apparently able to precipitate negatively charged colloids at reactions much more alkaline than this. There is also no proof at present that the protein concerned is actually produced by the liver, although this seems very probable. Notwithstanding

this uncertainty as to the mechanism, most workers who have used the tests have found them of value in the detection of liver damage.

Experimental.

The present communication is concerned with the serum colloidal gold reaction. There were certain difficulties in Gray's (1940) method which called for the standardization of each batch of sol with normal serum, using ten tubes with a serial dilution technique starting with serum diluted 350 times. According to Mateer *et al.* (1942) it is necessary to use at least 20 normal sera for each standardization, and to repeat this process every two weeks if false positives are to be avoided.

In the course of work directed towards simplifying this technique it soon became evident that it was desirable to exercise a rigorous control of the reaction of the medium with buffer solutions. When this was done the results became much more readily reproducible, and were largely independent of the initial pH of the gold sol. Table I shows a representative experiment on two typical sera. The method adopted was to add 0·1 ml. of serum to 0·9 ml. of buffer, mix, and then transfer 0·1 ml. to the next tube containing 0·9 ml. of buffer and so on. The volume of serum in the successive tubes was therefore approximately :

$$10^{-1},\ 10^{-2},\ 10^{-3},\ 10^{-4},\ 10^{-5},\ 10^{-6},\ \text{and}\ 10^{-7}\ \text{ml.}$$

2·5 ml. of gold sol were then added to each tube, and the results read after shaking and allowing to stand overnight, using the usual Lange notation in which 5 = complete precipitation of the gold sol.

TABLE I.—*Precipitation of 2·5 ml. of Gold Sol by Serial Dilutions of Serum.*

Buffer (0·9 ml.).	pH.	Ionic strength before addition of sol.	Normal. ml. of serum.							Cirrhosis. ml. of serum.						
			10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.
*Phosphate (Sorenson)	6·0	0·072	5	5	5	5	5	4	0	5	5	5	5	5	5	0
	6·5	0·096	5	0	0	4	5	5	0	5	5	5	5	5	5	0
	7·0	0·127	2	0	0	3	4	5	0	5	5	4	5	5	5	0
	7·0	0·032	5	1	0	1	1	0	0	5	5	5	5	5	5	0
*Barbitone HCl	7·0	0·04	5	0	0	0	0	0	0	5	5	5	5	5	5	0
	7·5	0·04	3	0	0	0	0	0	0	5	5	4	0	0	0	0
	8·0	0·04	0	0	0	0	0	0	0	5	4	0	0	0	0	0

* Prepared as described by Britton (1942).

The following conclusions appear to follow from these experiments :

(1) At pH 6·5 to 7·0 zone effects appear with normal serum. Experiments to be reported elsewhere suggest that this is probably due to precipitation of the gold by the globulins in the lower dilutions (10^{-1} ml.) and by the albumin in the higher dilutions (up to 10^{-6} ml.).

(2) At pH 6·0 the two zones merge, giving complete precipitation up to an extreme dilution (10^{-6} ml.) with normal and pathological sera.

(3) With the barbitone buffer there are no zone effects, probably because the albumin is not active. This is also true of pathological sera, although these may cause precipitation in amounts as low as 10^{-6} ml. at pH 7·0. Raising the pH above 7·0 diminishes the sensitivity of the reaction.

(4) Comparison of the three buffers at 7·0 indicates that increasing the ionic strength inhibits the precipitation of the sol by the globulin fraction while augmenting its precipitation by albumin, thus favouring the appearance of zone effects. For this

reason the barbitone buffer is to be preferred to the phosphate buffer, which has in any case rather feeble buffering power at an ionic strength of 0·04 owing to its divalent anion HPO_4.

On the basis of these experiments the following technique was finally chosen, and is designed to avoid zone effects and to use convenient volumes of serum (0·05 ml.) and buffer (0·5 ml.) at pH 7·8.

SOLUTIONS.

(1) *Colloidal gold sol* prepared by Patterson's (1931) oxalate method with slight modifications. The sol was made up in 500 ml. (not 1000 ml.) portions, and it was found to be essential for the mixture to reach the boiling-point in 6–7 minutes, necessitating the use of three bunsen burners. The preliminary titration was performed with 50 ml. portions (not 5 ml.), and these should boil in three minutes. The usual criteria of the slight sheen and the sequence of colour changes during heating were carefully adhered to. About 4 litres were usually prepared at one time, and the sol was found to keep without deterioration for periods up to 6 months if stored in Winchester quart bottles which had previously contained strong acids. It is, of course, not necessary to standardize the sol in any way as would be done for the Lange cerebrospinal fluid test, as the final reaction is controlled by the buffer solution. It is important *not* to pipette the sol direct from the stock bottle.

(2) *Barbitone buffer, pH* 7·8, $\mu = 0{\cdot}02$.—0·552 g. of barbitone (di-ethyl barbituric acid), 0·412 g. of sodium barbitone, and 0·2 g. of phenol are dissolved in 100 ml. of water with the aid of heat. This solution keeps for at least 2–3 months, the phenol serving to prevent the growth of moulds.

METHOD.

Measure 0·05 ml. of serum into a dry test-tube 4 in. by ½ in., using a 0·2 ml. pipette with a waxed tip. Add 0·5 ml. of buffer and then 2·5 ml. of gold sol. The buffer must be added first. The final ionic strength is about 0·01 including serum electrolytes and gold sol. Shake and allow to stand overnight. Record results as 5 = complete precipitation, 4 = supernatant fluid just coloured, 1 = gold precipitate just visible on shaking, 2 and 3 being intermediate grades of precipitation, judged mainly by the colour of the supernatant fluid. There is no change in tint as in the Lange test, but positive results are usually indicated by a turbidity appearing within 5–10 minutes.

The test can be made semi-quantitative by adding 0·1 ml. of serum to 0·9 ml. of buffer, mixing and transferring 0·5 ml. to the next tube containing 0·5 ml. of buffer and so on. Six tubes are usually sufficient, complete precipitation being rare after the fourth tube (0·00625 ml. of serum). This method was not employed extensively, but is useful in following the course of infective hepatitis. One typical case, e.g., gave the following results at weekly intervals:

555400 (serum bilirubin 6·5 mg. %)
552000 (,, ,, 2·9 ,,)
530000 (,, ,, 1·5 ,,)
300000 (,, ,, 0·9 ,,)
000000 (,, ,, 0·7 ,,)

Serum for the test was found to keep for several weeks in the refrigerator without change, and slight degrees of haemolysis do not interfere. Heating to 56° for 30 minutes partially abolishes the reaction, so that inactivated sera cannot be used.

Reproducibility.

The general reproducibility of the method was tested in two ways. Firstly, whenever a new batch of sol or buffer was prepared, a number of sera known to give positive and negative results with the old reagent were tested against the new one. No appreciable differences were found in this way. Secondly a suitable positive serum was tested against six different batches of sol of ages varying from a few days to six months, and they all gave identical degrees of precipitation (Grade 3). It appears therefore that these sols are reproducible and can be kept at least six months. In view of the work of Glasoe and Sorum (1939) on the reciprocal effects of pH and particle size on the sensitivity of gold sols, this would suggest that Patterson's method gives sols of a fairly constant particle size. Different batches no doubt vary slightly in reaction, but these variations do not influence the final reaction which is determined by the buffer.

RESULTS.

Table II shows the results in 200 cases of jaundice.

TABLE II.—*Serum Colloidal Gold Reaction in* 200 *Jaundiced Patients.*

Precipitation number.	5.	4.	3.	2.	1.	0.	Totals.
Obstructive	—	—	—	2	—	32	34
Infective hepatitis	63	11	13	9	4	5	105
Cirrhosis*	9	—	—	—	—	—	9
Arsenical	4	3	2	4	1	21	35
Weil's disease	—	—	4	2	3	8	17

* Only 5 of these were clinically jaundiced.

It appears from this that the test is usually positive in infective hepatitis (95 per cent.) and in hepatic cirrhosis (9 out of 9 cases). It may be either positive or negative in Weil's disease and in arsenical jaundice, the latter group being all patients undergoing anti-syphilitic treatment. It is almost uniformly negative in obstructive jaundice, the only two positive results here being in patients deeply jaundiced for three months.

TABLE III.—*Serum Colloidal Gold Reaction in* 200 *Non-jaundiced Patients.* 169 *Cases Negative,* 31 *Cases Positive.*

Each number gives the degree of precipitation in one Case.

A. Chronic debilitating diseases.	B. Acute virus infections.
Pulmonary tuberculosis : 3, 2, 1, 1.	—
Gross anaemia : 1, 1, 1, 1.	—
Heart failure : 5, 5, 3, 1.	Glandular fever : 3, 3, 1.
Urinary infection : 3, 2, 1, 1, 1.	Atypical pneumonia : 4, 2, 1. 1.
Syphilis : 2, 2, 2.	
Rheumatoid arthritis : 5, 4.	
Infective endocarditis : 5, 5.	

Table III shows the results in 200 non-jaundiced patients. Most of these were obtained by doing the test on every patient who was having any other chemical investigation done during a certain period, but some were also cases of known acute or chronic infections selected for that reason.

The 31 positive cases may be divided into two groups as in the table:

A. Conditions which have been called " chronic debilitating diseases " for want of a better term. These were all fairly ill patients, being either febrile or having some degree of cachexia.

B. Certain acute virus infections, possibly those with a special tendency to attack the liver. (Infective hepatitis is of course the most important member of this group.)

There is independent clinical or laboratory evidence of liver involvement in nearly all these conditions, so that it appears reasonable to assume that all the positive reactors had some degree of liver damage.

The 169 negative cases cover all the common medical and surgical conditions, including many examples of those occurring in the positive group, e.g. 21 cases of pulmonary tuberculosis. Evidently only a proportion of such patients give positive results. The absence of positive findings in any subject without definite constitutional disturbance appears to provide an adequate series of normal controls.

DISCUSSION.

Gray's (1940) serum colloidal gold test gave positive results in 100 per cent. of cases of cirrhosis, and in a high proportion of other cases with generalized liver damage. It was also frequently positive in obstructive jaundice and in cholelithiasis and gave 10 per cent. of positives in normal subjects (Mateer *et al.*, 1942). It is evident that the modification presented here gives results substantially different from these, probably because of the more alkaline reaction employed—pH 7·8 as opposed to Gray's value, which was stated to be in the neighbourhood of 7·0. The principal differences are that normal subjects now give uniformly negative results and cases of obstructive jaundice are almost uniformly negative, at least during the first three months ; on the other hand, the sensitivity of the reaction in infective hepatitis and in cirrhosis appears to be unimpaired. The modified test is simpler to perform than the old one and is well adapted to routine use.

The results as a whole suggest that the test described here is a good index of liver damage in a variety of conditions, with certain curious exceptions, of which obstructive jaundice and arsenical jaundice are the most important. There can be no doubt on clinical grounds that many of the negative reactors in these groups had severe degrees of hepatic dysfunction, and some of the obstructive cases showed considerable amounts of liver destruction—cholangitis, metastases, etc.—at autopsy. These considerations indicate that there may be qualitative differences in the various types of liver injury, and recall the rather similar results obtained with the cephalin-cholesterol flocculation test. Thus Nadler and Butler (1942) recorded mainly negative results in biliary obstruction, and Hanger and Gutman (1940) encountered negative reactors in arsenical jaundice. Pohle and Stewart (1941) on the other hand got positive results in obstructive jaundice, and there appears to be some confusion in this test between the use of ripened and unripened cephalin (Mateer *et al.*, 1942).

Apart from its value as a general indicator of liver dysfunction the serum colloidal gold reaction has proved useful in the investigation of jaundice of unknown origin. It will be seen from Table II that if the diagnosis lies between biliary obstruction on the one hand and infective hepatitis or cirrhosis on the other, the test provides a good prospect of differentiation, a negative result favouring the former and a positive result one of the latter. In cases of this type a positive result is more helpful than a negative one, which does not absolutely exclude infective hepatitis. If the test is positive it is, of course, necessary to eliminate the more obvious " chronic debilitating diseases," but this can usually be done without difficulty on clinical grounds. When used in

this way the test has given promise of being better than any other at present available for making this particular distinction.

The marked difference between the infective hepatitis and the arsenical cases is in complete contrast to the very similar results given by most other liver function tests and by liver biopsy studies in these two groups. This is of some interest in relation to the current view as to their similar or identical nature, and suggests that there may be a real pathological difference in the two conditions.

SUMMARY.

(1) The serum colloidal gold reaction is greatly influenced by the reaction and by the ionic strength of the medium. The technique has therefore been modified by the introduction of a buffer solution, which permits the test to be performed in a single tube in a reproducible manner without preliminary standardization of the gold sol. The conditions chosen give a final serum dilution of 1 in 60, a pH of 7·8, and an ionic strength of 0·01.

(2) The results obtained in 200 jaundiced and 200 non-jaundiced patients suggest that the test is a valuable indicator of liver damage. The almost uniformly negative results in obstructive jaundice give it a particular value in distinguishing this condition from infective hepatitis or hepatic cirrhosis.

(3) The results in arsenical jaundice (40 per cent. positive) were strikingly different from those in infective hepatitis (95 per cent. positive), suggesting some essential pathological difference in the two groups.

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COMPARISON BETWEEN THE REACTIONS OF ACUTE PHASE SERUM WITH PNEUMOCOCCUS C-POLYSACCHARIDE AND WITH PNEUMOCOCCUS TYPE 27.*

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In previous publications (Löfström, 1939, 1942, 1943) it was shown that a substance may occur in human serum which causes capsular swelling and agglutination of pneumococci of types 16, 27 and 28, and perhaps of other types as well. It is not a normal or an immune antibody and I have called it nonspecific capsular swelling substance. In this publication it is also called type 27 reactive substance by analogy with the C-reactive substance (Ash, 1933 ; Abernethy and Avery, 1941). It is formed in acute stages of bacterial disease, in some non-infectious acute diseases accompanied by disintegration of tissue and after the injection of antigens and non-specific stimulants. A substance of the same kind was also observed in rabbit serum ; it reacted regularly with pneumococcus type 16 but seldom and only weakly with types 27 and 28.

Tillett and Francis (1930) reported that precipitins against the somatic C-polysaccharide of pneumococcus occurred in sera from patients in the acute phase of an infectious disease. The further investigations of Tillett, Goebel and Avery (1930), Heidelberger and Kendall (1931), Ash (1933), Abernethy and Avery (1941), MacLeod and Avery (1941*a*, *b*) and of Perlman, Bullowa and Goodkind (1943) indicate that this C-reactive substance of acute phase serum agrees fairly well in behaviour and properties with the nonspecific capsular swelling substance.

Because of their great likenesses, I have suspected that the two substances are identical. In the present investigation the two are compared from new points of view, and a number of facts earlier considered to argue against their being identical are subjected to re-analysis.

METHODS.

Preparation of pneumococcus type 27 suspensions.

The pneumococcus type 27 suspensions were prepared with strain 27L obtained from the Danish State Serum Institute. It had been kept dried in a refrigerator and showed good capsular forming capacity. The bacteria were allowed to grow for six to eight hours on hormone broth to which 0·1 per cent. glucose had been added, and were killed by treatment with a 2 per cent. solution of commercial formol for 18 hours at room temperature. After centrifuging, the bacteria were suspended in physiologic salt solution containing 0·5 per cent. commercial formol.

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Preparation of pneumococcus C-polysaccharide.

Sixteen litres of hormone broth were seeded with type 1 pneumococci. The culture was allowed to grow for 24 hours, after which the bacteria were separated away and suspended in a small amount of physiologic salt solution. The suspension was frozen and thawed so as to obtain a thick, relatively homogeneous solution. After adding glacial acetic acid to bring the pH to about 3·4, the suspension was put in a boiling water bath for 20 minutes. The clot was removed by centrifugation and 5 volumes of 96 per cent. alcohol were added to the solution, which was then left to stand overnight in the refrigerator. The mixture was centrifuged and the precipitate dissolved in physiologic salt solution. The two precipitating processes, at pH about 3·4 and with alcohol, were then repeated alternately until all the material precipitable with acid and heat was removed. The remaining specific type 1 polysaccharide was precipitated with a saturated barium hydroxide solution. The precipitate was centrifuged away and the supernatant fluid freed from barium. Two volumes of alcohol were then added and, after the mixture has stood overnight in the refrigerator, the precipitate was centrifuged away. Alcohol, amounting to three times the volume of the original supernatant fluid, was again added. The precipitate was dissolved in physiologic salt solution, and renewed precipitation was carried out with alcohol and redistilled alcohol. The final precipitate was washed with ether and dried to light white powder. The C-polysaccharide so produced caused a trace of reaction in specific type 1 rabbit serum, and produced a coarsely flocculant precipitate when mixed with acute phase serum. It reacted with serum containing a high content of acute phase substance up to a dilution of 1 : 1,280,000.

Titration Methods.

1. *Nonspecific capsular swelling substance* (type 27 reactive substance).

The slide method was used for demonstrating capsular swelling and agglutination. It was carried out according to the Neufeld method, as described by me (Löfström, 1937).

A normal loopful of bacterial suspension is placed on a slide and mixed with a loopful of serum, after which a loopful of Löffler's alkaline methylene-blue diluted 1 : 2 is added for staining. A cover-glass is put on and half an hour later the preparation is ready for observation under the microscope. An immersion lens and magnification of at least one thousand times are used. Two phenomena are looked for in the microscope: firstly, capsular swelling which generally occurs instantaneously, and secondly, agglutination which often requires a certain length of time to become stabilized.

The titration of serum was done with a constant amount of serum against increasing bacterial concentrations. A suitable basic density for the suspension was found to correspond to a twentyfold dilution of Burroughs & Wellcome opacity tube No. 3, which gives a concentration of one to five bacteria per field of vision. This concentration was called density 1, and the corresponding content of non-specific capsular swelling substance titer 1. Densities 2 to 64 were obtained by successive doubling of the bacterial concentration in density 1.

2. *C-reactive substance.*

a. *Constant amount of serum against decreasing amounts of C-polysaccharide.*— 0·5 ml. of varying dilutions of precipitinogens were added to 0·25 ml. of serum diluted to 0·5 ml. with physiologic salt solution. Final readings were made after the mixtures had been kept in a water-bath at 50° C. for two hours and overnight in the refrigerator.

b. *Constant amount of C-polysaccharide against decreasing amounts of serum.*—0·5 ml. of varying dilutions of serum (1 : 2 – 1 : 32) was added to 0·5 ml. of a suitable dilution of C-polysaccharide (1 : 20,000). The mixtures were incubated and examined in the same way as in the foregoing method.

Earlier investigations on the C-reactive substance have been limited to demonstrating its occurrence in acute infectious diseases. In order to make a more all-round comparison of this substance with the type 27 reactive substance, the present investigation covers not only cases of acute pneumonia, but also ones of acute cardiac infarction and acute conditions produced by injections of a colloidal sulphur compound. The compound used was "Sulfosin," which contains 0·1 g. of sulphur per millilitre of oil. The C-reactive substance was titrated both with a constant amount of serum against decreasing amounts of antigen and with a constant amount of polysaccharide against decreasing amounts of serum. Table I covers the results of this study.

TABLE I.—*Testing of Somatic C-polysaccharide of Pneumococcus and of Pneumococcus Type 27 Against Sera from Cases in the Acute Stages of Disease.* (*N* = *negative.*)

Case number.	Diagnosis.	Day of disease.	Temperature (°C.).	P. type 27 titre.	Sera tested against C-polysaccharide.	
					Method 2a titre.	Method 2b titre.
1	Acute pneumonia	10	37·8	4	N	..
		14	38·0	8	1/80,000	1/4
2	" " + ac. polyarthritis	22	38·6	16	1/320,000	1/16
3	Acute pneumonia Type 1	27	37·7	8	1/320,000	1/8
4	Acute pneumonia Type 19	4	37·9	32	1/640,000	1/16
5	Acute pneumonia Type 5	5	37·6	8	1/320,000	1/16
6	Acute pneumonia	3	39·1	16	1/320,000	1/32
		10	37·2	N	N	..
7	" " Type 10	3	39·6	32	1/640,000	1/16
		14	37·1	N	N	..
8	Acute pneumonia Type 14	3	36·8	16	1/640,000	1/16
		13	36·8	N	N	..
9	Acute pneumonia	21	37·8	4	N	..
10	" "	7	37·2	1	N	..
11	Myocardial infarct	5	39·3	16	1/320,000	1/16
12	Sulfosin fever	2	38·0	8	1/640,000	1/8
13	" "	2	38·7	8	1/640,000	1/16

The experiments show that the C-polysaccharide reacted with acute phase serum from patients with acute pneumonia, and also from patients acutely ill from conditions of non-infectious origin, such as acute cardiac infarction and fever after injections of sulphur. Thus the C-reactive substance occurs under the same conditions as the type 27 reactive substance. Sometimes the C-polysaccharide reaction was negative when there was a slight type 27 reaction. The two substances seem to occur in about the same quantities, as is best seen from a comparison of the type 27 titre and the titre of the C-reactive substance, as tested with method 2b. This method requires less serum than the method 2a, and the results of this method are easily comparable with those obtained from the titration of the type 27 reactive substance. From now on, therefore, I shall mostly use the 2b method for titrating the C-reactive substance.

The C-polysaccharide was also tested against specific anti-pneumococcal serum from rabbits. The serum had a titre of 1/160 against pneumococci of type 27, but did not react with the C-polysaccharide.

Fractioning of Acute Phase Serum with Ammonium Sulphate.

One of the differences formerly thought to exist between the C-protein and type 27 reactive substance was that they occurred in different fractions of serum. MacLeod and Avery (1941*a*, *b*) believed that the C-protein occurred in the albumin fraction, and my investigations indicated that the type 27 reactive substance belonged to a globulin fraction, probably α or β. Perlman, Bullowa and Goodkind (1943), however, have shown that the C-protein is an α-globulin. To see how this observation corresponds with the results of the salting-out experiments with ammonium sulphate, the following new experiments were done.

Method for Fractional Salting-out with Ammonium Sulphate.

7·5 ml. of acute phase serum were diluted with an equal amount of distilled water and 15 ml. of saturated ammonium sulphate solution added. After two hours at 37° C. the globulin was centrifuged away and washed twice with a semisaturated solution of ammonium sulphate. It was then dissolved in physiologic salt solution, re-precipitated, washed again and dissolved in a small amount of physiologic salt solution. After dialysis overnight in a refrigerator against physiologic salt solution containing 0·025 per cent. calcium chloride, the volume was adjusted to 7·5 ml.

The albumin fraction was mixed with the first washing water from the globulin precipitation, raising the volume to 44 ml. Twenty-two ml. of saturated ammonium sulphate solution was added, bringing the final concentration of sulphate to 75 per cent. After an hour at room temperature the precipitate was removed by filtration through paper and dissolved in a small amount of physiologic salt solution. After dialysis according to the same method as for the globulin fraction, the volume was adjusted to 7·5 ml.

Table II shows the distribution of the reactive substances in the globulin and albumin fractions.

TABLE II.—*The Content of C- and Type 27 Reactive Substance in Different Fractions of Acute Phase Serum.*

Content of C-reactive substance: +++ = large, distinct floccules which gradually sink to the bottom of the test-tube; ++ = slightly smaller but distinct floccules; + = small floccules, easily observed with a magnifying glass; N = negative.

Case number.	Fractions.	Reacting polysaccharides. P. type 27.	C-polysaccharide diluted 1 : 40,000.
16	Globulin (semisaturation with $(NH_4)_2SO_4$)	1	N
	Albumin (50–75% saturation with $(NH4)_2SO_4$)	4	+++
	Untreated serum	8	+++
17	Globulin (semisaturation with $(NH_4)_2SO_4$)	N	N
	Albumin (50–75% saturation with $(NH4)_2SO_4$)	8	+++
	Untreated serum	16	+++

It appears from the table that the C-polysaccharide reacts with the albumin fraction obtained with 50 to 75 per cent. saturation with ammonium sulphate but not with the globulin fraction, which agrees with the observations of MacLeod and Avery (1941*a*, *b*). Type 27 also reacted almost exclusively with this albumin fraction, but traces of reactive substance also seemed to occur in the globulin.

Absorption Tests.

Cross absorption with C-polysaccharide and type 27 pneumococci and certain other pneumococci was carried out to settle the question whether the active substances occurring in acute phase serum are identical.

Methods.

Absorption with bacteria.—One millilitre of a bacterial suspension with a density corresponding to twice that of the reactive 27 substance was mixed with one millilitre of acute phase serum. One hour at room temperature was allowed for binding.

Absorption with C-polysaccharide.—One millilitre of a C-polysaccharide solution diluted 1 : 10,000 was mixed with one millilitre of acute phase serum. One hour at room temperature was allowed for binding.

Table III gives the results of the absorption experiments.

TABLE III.—*Absorption of Acute Phase Serum with Pneumococcus Type 27 and C-polysaccharide.*

A 27, A 1, A β and A C denote sera after absorption with bacterial suspensions of pneumococcus type 27, pneumococcus type 1 and beta streptococci and with C-polysaccharide in dilution of 1 : 10,000. N = negative.

Case number.	Bacterial density used in absorption.	Reacting P. 27 or C-polysaccharide.	Untreated serum.	Test after absorption.			
				A27.	A1.	Aβ.	AC.
18	16	27	8	N	8	4	N
		C 1 : 40,000	+++	N	+++	+++	−
19	32	27	16	N	2	8	N
		C 1 : 40,000	+++	N	+	+++	N

The experiments reveal that complete cross-absorption took place of the reactive substances against pneumococcus type 27 and the C-polysaccharide. Bacteria like pneumococcus type 1 and haemolytic streptococci had hardly any influence on the serologic activity in acute phase serum.

DISCUSSION.

It has been found on comparison between the reactions of acute phase serum with the somatic C-polysaccharide of pneumococci and with pneumococci of type 27 that the reactive substances are alike both in behaviour and properties. The complete cross-absorption, in particular, seems to indicate that the two substances are identical. If the results of all the research on this field are combined, the reactive acute phase substance is seen to have the following properties: It is a protein, and is precipitated in the presence of calcium by the somatic C-polysaccharide of pneumococci. Under similar conditions it causes capsular swelling of pneumococci of types 16, 27 and 28, and perhaps of other types as well. It is not a normal or an immune antibody. It is formed in acute stages of bacterial disease, in some non-infectious acute diseases

accompanied by disintegration of tissue and after the injection of antigens and non-specific stimulants. The concentration of the reactive protein in serum is low in comparison to that of immune antibodies. It has good antigenic properties and produces a potent anti-" reactive protein " serum in rabbits.

The reason for the connection between the C-polysaccharide and the reacting capsular substances is obscure. Since the C-polysaccharide is not precipitated by specific type 27 antipneumococcic serum from rabbits, it is hardly likely that the specific polysaccharides of the pneumococci in question react with the acute phase serum. Perhaps the capsules of the reacting pneumococcic types contain C-polysaccharides or closely related polysaccharides in addition to specific polysaccharides.

It is not yet known how the reactive acute phase protein develops. Since it occurs in conditions where infection can be ruled out, its reaction with bacterial substances of antigenic nature can hardly have any special causal connection with the reaction of the organism against bacterial products. It seems more likely, from the fact that local injections of sulphur stimulate the development of acute phase protein, that the disintegration of tissue at the site of injection gives rise to products which, by being absorbed into the blood, cause a stimulation of the globulin building apparatus. The nature of these hypothetical disintegration products is not known.

SUMMARY.

The reaction between the somatic C-polysaccharide of pneumococci and serum from cases of acute infectious disease, first observed by Tillett and Francis (1930), was compared with the non-specific capsular swelling reaction (Löfström). The reactive substances, the C-reactive protein and the type 27 reactive protein, agree as regards behaviour and properties, and their identity is confirmed by tests with cross-absorption. The acute phase protein is formed in acute stages of bacterial disease, in some non-infectious acute diseases accompanied by disintegration of tissue, and after the injection of non-specific stimulants such as colloidal sulphur.

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